Polyketides and their Synthesis

The present invention relates to processes and
materials (including enzyme systems, nucleic acids, vectors
5   and cultures) for preparing 14-membered macrolides by
recombinant synthesis and to the novel polyketides so
produced.   Polyketide biosynthetic genes or portions of
them, which may be derived from different polyketide
biosynthetic gene clusters are manipulated to allow the
10 production of specific novel polyketides, such as 12-, 14-
and 16-membered macrolides, of predicted structure. This
invention is particularly concerned with the replacement of
genetic material encoding the natural starter unit with
other genes in order to prepare 14-membered macrolides with
15 preferentially an acetate starter unit, whilst minimising
the formation of by-products containing a different starter
unit.

Polyketides are a large and structurally diverse class
of natural products that includes many compounds possessing
20 antibiotic or other pharmacological properties, such as
erythromycin, tetracyclines, rapamycin, avermectin,
monensin, epothilones and FK506. In particular,
polyketides are abundantly produced by *Streptomyces* and
related actinomycete bacteria. They are synthesised by the
25 repeated stepwise condensation of acylthioesters in a
manner analogous to that of fatty acid biosynthesis. The
greater structural diversity found among natural
polyketides arises from the selection of (usually) acetate
or propionate as starter or "extender" units; and from the

differing degree of processing of the β-keto group observed after each condensation. Examples of processing steps include reduction to β-hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of chain extension.

The biosynthesis of polyketides is initiated by a group of chain-forming enzymes known as polyketide synthases. Two classes of polyketide synthase (PKS) have been described in actinomycetes. One class, named Type I PKSs, represented by the PKSs for the macrolides erythromycin, oleandomycin, avermectin and rapamycin, consists of a different set or "module" of enzymes for each cycle of polyketide chain extension. For an example see Figure 1 (Cortés, J. et al. Nature (1990) 348:176-178; Donadio, S. et al. Science (1991) 2523:675-679; Swan, D.G. et al. Mol. Gen. Genet. (1994) 242:358-362; MacNeil, D. J. et al. Gene (1992) 115:119-125; Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843).

The term "extension module" as used herein refers to the set of contiguous domains, from a β-ketoacyl-ACP synthase ("AKS") domain to the next acyl carrier protein ("ACP") domain, which accomplishes one cycle of polyketide chain extension. The term "loading module" is used to refer to any group of contiguous domains which accomplishes the loading of the starter unit onto the PKS and thus renders it available to the KS domain of the first

extension module. The length of polyketide formed has been altered, in the case of erythromycin biosynthesis, by specific relocation using genetic engineering of the enzymatic domain of the erythromycin-producing_PKS that

5   contains the chain releasing thioesterase/cyclase activity (Cortés *et al.* Science (1995) 268:1487-1489; Kao, C.M. *et al.* J. Am. Chem. Soc. (1995) 117:9105-9106).

In-frame deletion of the DNA encoding part of the ketoreductase domain in module 5 of the erythromycin-

10 producing PKS (also known as 6-deoxyerythronolide B synthase, DEBS) has been shown to lead to the formation of erythromycin analogues 5,6-dideoxy-3-α-mycarosyl-5-oxoerythronolide B, 5,6-dideoxy-5-oxoerythronolide B and 5,6-dideoxy, 6 β-epoxy-5-oxoerythronolide B (Donadio, S. *et*

15 *al.* Science (1991) 252:675-679). Likewise, alteration of active site residues in the enoylreductase domain of module 4 in DEBS, by genetic engineering of the corresponding PKS-encoding DNA and its introduction into *Saccharopolyspora erythraea*, led to the production of 6,7-anhydroerythromycin

20 C (Donadio, S. *et al.* Proc Natl. Acad. Sci. USA (1993) 90:7119-7123).

International Patent Application number WO 93/13663 describes additional types of genetic manipulation of the DEBS genes that are capable of producing altered

25 polyketides. However many such attempts are reported to have been unproductive (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238, at p. 231). The complete DNA sequence of the genes from *Streptomyces*

hygroscopicus that encode the modular Type I PKS governing the biosynthesis of the macrocyclic immunosuppressant polyketide rapamycin has been disclosed (Schwecke, T. *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843). The

5   DNA sequence is deposited in the EMBL/Genbank Database under the accession number X86780.

The second class of PKS, named Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs contain only a single set of enzymatic activities

10 for chain extension and these are re-used as appropriate in successive cycles (Bibb, M. J. *et al.* EMBO J. (1989) 8:2727-2736; Sherman, D. H. *et al.* EMBO J. (1989) 8:2717-2725; Fernandez-Moreno, M.A. *et al.* J. Biol. Chem. (1992) 267:19278-19290). The "extender" units for the Type II

15 pKSs are usually acetate units, and the presence of specific cyclases dictates the preferred pathway for cyclisation of the completed chain into an aromatic product (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238). Hybrid polyketides have been obtained

20 by the introduction of clones Type II PKS gene-containing DNA into another strain containing a different Type II PKS gene cluster, for example by introduction of DNA derived from the gene cluster for actinorhodin, a blue-pigmented polyketide from *Streptomyces coelicolor*, into an

25 anthraquinone polyketide-producing strain of *Streptomyces galileus* (Bartel, P. L. *et al.* J. Bacteriol. (1990) 172:4816-4826).

The minimal number of domains required for polyketide

chain extension on a Type II PKS when expressed in a
Streptomyces coelicolor host cell (the "minimal PKS") has
been defined for example in International Patent
Application Number WO 95/08548 as containing the following

5   three polypeptides which are products of the *act I* genes:
first KS; secondly a polypeptide termed the CLF with end-
to-end amino acid sequence similarity to the KS but in
which the essential active site residue of the KS, namely a
cysteine residue, is substituted either by a glutamine

10   residue, or in the case of the PKS for a spore pigment such
as the *whiE* gene product (Chater, K. F. and Davis, N. K.
Mol. Microbiol. (1990) 4:1679-1691) by a glutamic acid
residue (Figure 2); and finally an ACP. The CLF has been
stated for example in International Patent Application

15   Number WO 95/08548 to be a factor that determines the chain
length of the polyketide chain that is produced by the
minimal PKS. However it has been found (Shen, B. *et al.* J.
Am. Chem. Soc. (1995) 117:6811-6821) that when the CLF for
the octaketide actinorhodin is used to replace the CLF for

20   the decaketide tetracenomycin in host cells of *Streptomyces
glaucescens*, the polyketide product is not found to be
altered from a decaketide to an octaketide, so the exact
role of the CLF remains unclear. An alternative
nomenclature has been proposed in which KS is designated

25   KSα and CLF is designated KSβ, to reflect this lack of
knowledge (Meurer, G. *et al.* Chemistry and Biology (1997)
4:433-443). The mechanism by which acetate starter units
and acetate extender units are loaded onto the Type II PKS

is not known, but it is speculated that the malonyl-CoA: ACP acyltransferase of the fatty acid synthase of the host cell can fulfil the same function for the Type II PKS (Revill, W. P. *et al.* J. Bacteriol. (1995) 177:3946-3952).

5   International Patent Application Number WO 95/08548 describes the replacement of actinorhodin PKS genes by heterologous DNA from other Type II PKS gene clusters, to obtain hybrid polyketides. The same International Patent Application WO 95/08548 describes the construction of a

10   strain of *Streptomyces coelicolor* which substantially lacks the native gene cluster for actinorhodin, and the use in that strain of a plasmid vector pRM5 derived from the low-copy number vector SCP2* isolated from *Streptomyces coelicolor* (Bibb, M. J. and Hopwood, D. A. J. Gen.

15   Microbiol. (1981) 126:427) and in which heterologous PKS-encoding DNA may be expressed under the control of the divergent *act I/ act* III promoter region of the actinorhodin gene cluster (Fernandez-Moreno, M.A. *et al.* J. Biol. Chem. (1992) 267:19278-19290). The plasmid pRM5 also

20   contains DNA from the actinorhodin biosynthetic gene cluster encoding the gene for a specific activator protein, ActII-orf4. The Act II-orf4 protein is required for transcription of the genes placed under the control of the actI/ act II bidirectional promoter and activates gene

25   expression during the transition from growth to stationary phase in the vegetative mycelium (Hallam, S. E. *et al.* Gene (1988) 74:305-320).

Type II clusters in *Streptomyces* are known to be

activated by pathway-specific activator genes (Narva, K. E. and Feitelson, J. S. J. Bacteriol. (1990) 172:326-333; Stutzman-Engwall, K. J. *et al.* J. Bacteriol. (1992) 174:144-154; Fernandez-Moreno, M.A. *et al.* Cell (1991) 66:769-780; Takano, E. *et al.* Mol. Microbiol. (1992) 6:2797-2804; Takano, E. *et al.* Mol. Microbiol. (1992) 7:837-845), The DnrI gene product complements a mutation in the *actII*-orf4 gene of *S. coelicolor*, implying that DnrI and ActII-orf4 proteins act on similar targets. A gene (*srmR*) has been described (EP 0 524 832 A2) that is located near the Type I PKS gene cluster for the macrolide polyketide spiramycin. This gene specifically activates the production of the macrolide antibiotic spiramycin, but no other examples have been found of such a gene. Also, no homologues of the ActII-orf4/DnrI/RedD family of activators have been described that act on Type I PKS genes.

Although large numbers of therapeutically important polyketides have been identified, there remains a need to obtain novel polyketides that have enhanced properties or possess completely novel bioactivity. The complex polyketides produced by Type I PKSs are particularly valuable, in that they include compounds with known utility as anthelminthics, insecticides, immunosuppressants, antifungal or antibacterial agents. Because of their structural complexity, such novel polyketides are not readily obtainable by total chemical synthesis, or by chemical modifications of known polyketides.

There is also a need to develop reliable and specific ways of deploying individual modules in practice so that all, or a large fraction, of hybrid PKS genes that are constructed, are viable and produce the desired polyketide

5   product.

Pending International Patent Application number PCT/GB97/01819 discloses that a PKS gene assembly (particularly of Type I) encodes a loading module which is followed by at least one extension module. Thus Figure 1

10   shows the organisation of the DEBS genes. The first open reading frame encodes the first multi-enzyme or cassette (DEBS 1) which consists of three modules: the loading module (ery-load) and two extension modules (modules 1 and 2). The loading module comprises an acyltransferase and an

15   acyl carrier protein. This may be contrasted with Fig. 1 of WO 93/13663 (referred to above). This shows ORF1 to consist of only two modules, the first of which is in fact both the loading module and the first extension module.

PCT/GB97/01819 describes in general terms the

20   production of a hybrid PKS gene assembly comprising a loading module and at least one extension module. PCT/GB97/01819 also describes (see also Marsden, A. F. A. et al. Science (1998) 279:199-202) construction of a hybrid PKS gene assembly by grafting the wide-specificity loading

25   module for the avermectin-producing polyketide synthase onto the first multienzyme component (DEBS 1) for the erythromycin PKS in place of the normal loading module. Certain novel polyketides can be prepared using the hybrid PKS gene assembly, as described for example in pending

International Patent Application number (PCT/GB97/01810).
Patent Application PCT/GB97/01819 further describes the
construction of a hybrid PKS gene assembly by grafting the
loading module for the rapamycin-producing polyketide

5   synthase onto the first multienzyme component (DEBS 1) for
the erythromycin PKS in place of the normal loading module.
The loading module of the rapamycin PKS differs from the
loading modules of DEBS and the avermectin PKS in that it
comprises a CoA ligase domain, an enoylreductase ("ER")

10   domain and an ACP, so that suitable organic acids including
the natural starter unit 3,4-dihydroxycyclohexane
carboxylic acid may be activated in situ on the PKS loading
domain, and with or without reduction by the ER domain
transferred to the ACP for intramolecular loading of the KS

15   of extension module 1 (Schwecke, T. et al. Proc. Natl.

Acad. Sci. USA (1995) 92:7839-7843).

The DNA sequences have been disclosed for several Type
I PKS gene clusters that govern the production of 16-
membered macrolide polyketides, including the tylosin PKS

20   from Streptomyces fradiae (EP 0 791 655 A2), the

niddamycin PKS from Streptomyces caelestis (Kavakas, S. J.

et al. J. Bacteriol. (1998) 179:7515-7522) and the

spiramycin PKS from Streptomyces ambofaciens (EP 0791 655

A2). All of these gene sequences have in common that they

25   show the loading module of the PKS to differ from the
loading module of DEBS and of the avermectin PKS in that
they consist of a domain resembling the KS domains of the
extension modules, an AT domain and an ACP (Figure 3). The
additional N-terminal KS-like domain has been named KSq

because it differs in each case from an extension KS by the specific replacement of the active site cysteine residue essential for β-ketoacyl-ACP synthase activity by a glutamine (Q in single letter notation) residue. The

5 function of the KSq domain is unknown (Kavakas, S. J. et al. J. Bacteriol. (1998) 179:7515-7522), but its presence in these PKSs for 16-membered macrolides is surprising because the starter units of tylosin, niddamycin and spiramycin appear to be propionate, acetate and acetate

10 respectively, that is, the same type of starter unit as in DEBS. The AT adjacent to the KSq domain is named here the ATq domain.

When the entire loading module of the tylosin PKS was used to replace the analogous loading module in the

15 spiramycin PKS in S. ambofaciens (Kuhstoss et al. Gene (1996) 183:231-236), the nature of the starting unit was stated to be altered from acetate to propionate. Since the role of the KSq domain was not understood, no specific disclosure was made that revealed either the importance of

20 the KSq domain, or the possible utility of these KSq-containing loading modules in ensuring the purity of the polyketide product in respect of the starter unit, even at high levels of macrolide production. The interpretation for their results was stated as: "Therefore we believe that the

25 experiments described here provide strong experimental support for the hypothesis that the AT domains in Type I PKS systems select the appropriate substrate at each step in synthesis" (Kuhstoss et al. Gene (1996) 183:231-236, at p. 235). These authors noted the analogy with the CLF

protein in Type II PKS systems and that the latter protein is thought to be involved in determining the chain length. They state: "KSq may serve a similar function, although it is unclear why such a function would be necessary in the
5   synthesis of these 16-membered polyketides when it is not needed for the synthesis of other complex polyketides such as 6-DEB or rapamycin. In any case the KSq is unlikely to be involved in substrate choice at each step of synthesis." (Kuhstoss *et al.* Gene (1996) 183:231-236).

10   It has been shown that when genetic engineering is used to remove the loading module of DEBS, the resulting truncated DEBS in *Sacch. erythraea* continues to produce low levels of erythromycins containing a propionate starter unit (Pereda, A. *et al.* Microbiology (1995) 144:543-553) .
15   The same publication shows that when in this truncated DEBS the methylmalonyl-CoA -specific AT of extension module 1 was replaced by a malonyl-CoA-specific AT from an extension module of the rapamycin PKS, the products were also low levels of erythromycins containing a propionate starter
20   unit, demonstrating that the origin of the starter units is not decarboxylation of the (methyl)malonyl groups loaded onto the enzyme by the AT of module 1, but from direct acylation of the KS of extension module 1 by propionyl-CoA. This is in contrast to a previous report, using partially
25   purified DEBS1+TE, a truncated bimodular PKS derived from DEBS (Kao, C. M. *et al.* J. Am. Chem. Soc. (1995) 117:9105-9106) and functionally equivalent to DEBS1-TE (Brown, M. J. B. *et al.*, J. Chem. Soc. Chem. Commun. (1995) 1517-1518;

Cortés, J. *et al.* Science (1991) 2523:675-679), which
stated that the origins of the starter units for DEBS can
include methylmalonate units which are loaded onto module 1
and are decarboxylated by the KS of module 1 (Pieper, R. *et*

5   *al.* Biochemistry (1997) 36:1846-1851). It has now been
found that when the DEBS1-TE protein is fully purified from
extracts of recombinant *Sacch. erythraea* it contains no
such specific decarboxylase activity (Weissmann, K. et al.
(1998) Biochemistry, 37, 11012-11017), further confirming

10   that starter units do not in fact arise from
decarboxylation of extension units mediated by the KS of
extension module 1 .

It is known that the DEBS loading module has a
slightly broader specificity than propionate only, and in

15   particular acetate starter units are used both in vitro and
in vivo, when the PKS containing this loading module is
part of a PKS that is expressed either in *Sacch. erythraea*
the natural host for erythromycin production (see for
example Cortés, J. *et al.* Science (1995) 268:1487-1489), or

20   in an heterologous host such as *S. coelicolor* (Kao, C. M.
et al. J. Am. Chem. Soc. (1994) 116:11612-11613; Brown, M.
J. B. *et al.* J. Chem. Soc. Chem. Commun. (1995) 1517-1519).
In vitro experiments using purified DEBS1-TE have
demonstrated that propionyl-CoA and acetyl-CoA are

25   alternative substrates that efficiently supply propionate
and acetate units respectively to the loading module
(Wiessmann, K. E. H. *et al.* Chemistry and Biology (1995)

2:583-589; Pieper, R. *et al.* J. Am. Chem. Soc. (1995)

117:11373-11374). The outcome of the competition between

acetate and propionate starter units is influenced by the

respective intracellular concentrations of propionyl-CoA

5   and acetyl-CoA prevailing in the host cell used (see for

example Kao, C. M. *et al.* Science (1994) 265:509-512;

Pereda, A. *et al.* Microbiology (1995) 144:543-553). It is

also determined by the level of expression of the host PKS,

so that as disclosed for example in Pending International

10   Patent Application number PCT/GB97/01819, when recombinant

DEBS or another hybrid PKS containing the DEBS loading

module is over-expressed in *Sacch. erythraea*, the products

are generally mixtures whose components differ only in the

presence of either an acetate or a propionate starter unit.

15

There is a need to develop reliable methods for

avoiding the formation of mixtures of polyketides with both

acetate and propionate starter units, and to allow the

specific incorporation of unusual starter units. It has now

20   been found, surprisingly, that the role of the loading

domains in the PKSs for the 16-membered macrolides tylosin,

niddamycin and spiramycin is different from that of the

loading domains of the avermectin PKS and of DEBS. It has

been realised that the KSq domain of the tylosin PKS and

25   the associated AT domain, which is named here ATq, together

are responsible for the highly specific production of

propionate starter units because the ATq is specific for

the loading of methylmalonyl-CoA and not propionyl-CoA as

previously thought; and the KSq is responsible for the

highly specific decarboxylation of the enzyme-bound
methylmalonate unit to form propionate unit attached to the
ACP domain of the loading module and appropriately placed
to be transferred to the KS of extension module 1 for the

5   initiation of chain extension. In a like manner the ATq of
the spiramycin and niddamycin PKSs, and the adjacent KSq,
are responsible for the specific loading of malonate units
rather than acetate units as previously believed, and for
their subsequent specific decarboxylation to provide

10   acetate starter units for polyketide chain extension.

It has also now been found here that not only the PKSs
for the above-mentioned 16-membered macrolides, but also
the PKSs for certain 14-membered macrolides particularly
the oleandomycin PKS from *Streptomyces antibioticus* (Figure

15   4) and also the PKSs for certain polyether ionophore
polyketides particularly the putative monensin PKS from
Streptomyces cinnamonensis (Figure 4), possess a loading

domain comprising a KSq domain, an ATq domain, and an ACP.
In Figure 4 is shown a sequence alignment of the KSq

20   domains and of the adjacent linked ATq domains that have
been identified, showing the conserved active site
glutamine (Q) residue in the KSq domains, and an arginine
residue which is conserved in all extension AT domains and
is also completely conserved in ATq domains. This residue

25   is characteristically not arginine in the AT domains of
either DEBS or of the avermectin PKS loading modules, where
the substrate for the AT is a non-carboxylated acyl-CoA
ester (Haydock, S. F. *et al.* FEBS Letters (1995) 374:246-

248) . The abbreviation ATq is used here to simply to

distinguish the AT domains found immediately C-terminal of Ksq from extension ATs, and the label has no other significance.

In one aspect this invention provides a PKS multienzyme or part thereof, or nucleic acid (generally DNA) encoding it, said multienzyme or part comprising a loading module and a plurality of extension modules for the generation of novel, 14-membered macrolides wherein

(a) the loading module is adapted to load a malonyl residue and then to effect decarboxylation of the loaded residue to provide an acetyl residue for transfer to an extension module; and

(b) the extension modules, or at least one thereof (preferably at least the one adjacent the loading module), are not naturally associated with a loading module that effects decarboxylation of an optionally substituted malonyl residue.

Generally the loading module will also include an ACP (acyl carrier protein) domain.

Preferably the decarboxylating functionality of the loading module is provided by a KS (ketosynthase)-type domain. Suitably this differs from a KS of a conventional extension module by possessing a glutamine residue in place of the essential cysteine residue in the active site. It is termed Ksq. It may be "natural" or genetically engineered, e.g. resulting from site-directed mutagenesis of nucleic acid encoding a different KS such as a KS of an extension module.

Alternatively the decarboxylating functionality can be provided by a CLF-type domain of the general type occuring in Type II PKS systems.

Preferably the loading functionality is provided by an AT (acyltransferase)-type domain which resembles an AT domain of a conventional extension module in having an arginine residue in the active site, which is not the case with the AT domains of loader modules which load acetate or propionate, e.g. in DEBS or avermectin PKS systems. It may be termed Atq. Once again, it may be "natural" or genetically engineered, e.g. by mutagenesis of an AT of an extension module.

Usually the loading module will be of the form:

Ksq-ATq-ACP

where ACP is acyl carrier protein.

In another aspect the invention provides a method of synthesising novel, 14-membered polyketides having substantially exclusively a desired acetate starter unit by providing a PKS multienzyme incorporating a loading module as defined above which specifically provides the desired acetate starter unit. This may comprise providing nucleic acid encoding the multienzyme and introducing it into an organism where it can be expressed.

In further aspects the invention provides vectors and transformant organisms and cultures containing nucleic acid encoding the multienzyme. A preferred embodiment is a culture which produces a 14-membered polyketide having a desired acetate starter unit characterised by the substantial absence of polyketides with different starter units. Thus, for example, C13-methyl-erythromycin can be

produced substantially free from natural analogues resulting from the incorporation of propionate starter units.

It is particularly useful to provide a loading module of the type KSq - ATq-ACP for a PKS gene assembly which produces a 14-membered macrolide in order to prepare a 14-membered macrolide which contains exclusively or almost exclusively an acetate starter unit, even when such PKS gene assembly is expressed at high levels in an actinomycete host cell. Particularly suitable PKSs for this purpose are the components of PKSs for the biosynthesis of erythromycin, methymycin, oleandomycin, tylosin, spiramycin, midecamycin, and niddamycin for all of which the gene and modular organisation is known at least in part. Particularly suitable sources of the genes encoding a loading module of the type KSq - ATq-ACP are the loading modules of oleandomycin, spiramycin, niddamycin, methymycin and monensin which are specific for the loading of malonate units which are then decarboxylated to acetate starter units.

In the loading module of the type KSq - ATq-ACP the domains or portions of them may be derived from the same or from different sources, and comprise either natural or engineered domains. For example the ATq domain can be replaced by an AT domain derived from any extension module of a Type I PKS, having specificity for loading of malonate units, so long as the KSq domain is chosen to have a matching specificity towards malonate units.

Alternatively, the KSq domain in the loading module provided of the type KSq - ATq-ACP may be substituted by

the CLF polypeptide of a Type II PKS. It is now apparent
that in contrast to its previous identification as a factor
uniquely determining chain length, the CLF, in addition to
any other activities that it may possess, is the analogue
of the KSq domain and can act as a decarboxylase towards
bound malonate units.

The appreciation that the CLF domain of Type II PKS's
has decarboxylating activity has led us to devise useful
interventions in Type II systems, e.g. to enhance the
yields obtainable in some fermentations. Many high-
yielding industrial fermentations tend to give mixtures,
owing to the incorporation of undesired starters. This is
particularly the case in systems which have auxiliary genes
for generating unusual starters. CLF genes may act to
produce undesired acyl species, leading to products
incorporating the undesired acyl units.

For example the production of oxytetracycline involves
an unusual malonamido starter. However the undesired
activity of a CLF domain causes some decarboxylation,
leading to the incorporation of acetyl instead. Daunomycin
synthesis likewise involves an unusual starter which is
liable to the "parasitic" activity of a CLF domain.

The active site (for decarboxylation) of a CLF domain
generally includes a glutamine residue. We find that the
decarboxylating activity of the domain can be removed by a
mutation by which the Gln residue is converted into (for
example) Ala.

Thus in a further aspect the invention provides a

system and process for synthesis of a type II (aromatic) polyketide, in which a gln residue of a CLF domain of the type II PKS is mutated to suppress decarboxylation activity. Techniques of site-specific mutagenesis by which
5 this can be achieved are by now well known to those skilled in the art.

The loading module of the type KSq - ATq-ACP may be linked to a hybrid PKS produced for example as in PCT/GB97/01819 and PCT/GB97/01810. It is particularly
10 useful to link such a loading module to gene assemblies that encode hybrid PKSs that produce novel derivatives of 14-membered macrolides as described for example in PCT/GB97/01819 and PCT/GB97/01810.

The invention further provides such PKS assemblies
15 furnished with a loading module of the type KSq - ATq-ACP, vectors containing such assemblies, and transformant organisms that can express them. Transformant organisms may harbour recombinant plasmids, or the plasmids may integrate. A plasmid with an *int* sequence will integrate
20 into a specific attachment site (*att*) of the host's chromosome. Transformant organisms may be capable of modifying the initial products, eg by carrying out all or some of the biosynthetic modifications normal in the production of erythromycins (as shown in Figure 5) and for
25 other polyketides. Use may be made of mutant organisms such that some of the normal pathways are blocked, e.g. to produce products without one or more "natural" hydroxy-groups or sugar groups. The invention further provides novel polyketides as producible, directly or indirectly, by

transformant organisms. This includes polyketides which have undergone enzymatic modification.

In a further aspect the invention provides both previously-obtained 14-membered ring macrolides and novel 14-membered ring macrolides in a purer form with respect to the nature of the acetate starter unit, than was hitherto possible. These include 14-membered ring macrolides which are either "natural" or may differ from the corresponding "natural" compound:

a) in the oxidation state of one or more of the ketide units (ie selection of alternatives from the group: -CO-, -CH(OH)-, alkene -CH-, and $-CH_2-$) where the stereochemistry of any -CH(OH)- is also independently selectable;

b) in the absence of a "natural" methyl side-chain; or

c) in the stereochemistry of "natural" methyl; and/or ring substituents other than methyl.

It is also possible to prepare derivatives of 14-membered ring macrolides having the differences from the natural product identified in two or more of items a) to c) above.

Derivatives of any of the afore-mentioned polyketides which have undergone further processing by non-PKS enzymes, eg one or more of hydroxylation, epoxidation, glycosylation and methylation may also be prepared.

The present invention provides a novel method of obtaining both known and novel complex 14-membered macrolides having an acetate starter unit substantively

free of products differing only in having a propionate starter unit.

Suitable plasmid vectors and genetically engineered cells suitable for expression of PKS genes incorporating an altered loading module are those described in PCT/GB97/01819 as being suitable for expression of hybrid PKS genes of Type I. Examples of effective hosts are *Saccharopolyspora erythraea*, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseofuscus*, *Streptomyces cinnamonensis*, *Streptomyces fradiae*, *Streptomyces longisporoflavus*, *Streptomyces hygroscopicus*, *Micromonospora griseorubida*, *Streptomyces lasaliensis*, *Streptomyces venezuelae*, *Streptomyces antibioticus*, *Streptomyces lividans*, *Streptomyces rimosus*, *Streptomyces albus*, *Amycolatopsis mediterranei*, and *Streptomyces tsukubaensis*. These include hosts in which SCP2*-derived plasmids are known to replicate autonomously, such as for example *S. coelicolor*, *S. avermitilis* and *S. griseofuscus*; and other hosts such as *Saccharopolyspora erythraea* in which SCP2*-derived plasmids become integrated into the chromosome through homologous recombination between sequences on the plasmid insert and on the chromosome; and all such vectors which are integratively transformed by suicide plasmid vectors.

Although some 13-methyl erythromycins (also known as 15-norerythromycins) have been reported previously (Kibwage et

al., J. Antibiotics, **40**, 1-6, 1987; Weber & McAlpine, U.S.

Patent 5,141,926), these have been confined to 15-

norerythromycin C, and 6-deoxy-15-norerythromycins B and D.

Moreover, not only have these 15-norerythromycins been

5   found as extremely minor components co-expressed with high

levels of "natural" erythromycins (13-ethyl erythromycins),

but the 13-methyl counterparts (15-norerythromycins A and

B) to the most desirable and biologically-active "natural"

erythromycins (erythromycin A and B) have never been

10   previously isolated. Chemical modification of "natural"

erythromycins has proven to be an extremely effective means

for enhancing the bioefficacy of the "natural" molecules.

Thus, it would be envisaged that chemical modification of

novel erythromycins would similarly produce compounds with

15   desirable and enhanced bioefficacies. PCT/GB97/01819

describes in general terms the production of novel

polyketides through recombinant DNA technologies, and the

use of these technologies to generate novel erythromycins,

many of which have different starter units to the

20   propionate starter unit characteristic of the "natural"

erythromycins, are described in pending International

Patent Application PCT/GB97/01810, Some chemical

modification of these novel erythromycins are also

described in co-pending International Patent Applications

25   PCT/IB98/02100 and PCT/IB98/02099. However, it is clear that the

ability to produce novel erythromycins at good expression

levels and in the substantial absence of novel or natural

erythromycins with different starter units is essential to facilitate the ability to achieve a wide range of chemical modifications to such novel erythromycins. The enhanced ability to produce polyketides at good expression levels

5   and in the substantial absence of polyketides with different starter units has been described in this application are family members, and we now describe the ability to produce 13-methyl erythromycins at good expression levels and in the substantial absence of

10   erythromycins with different starter units. The use of this technology has now permitted the preparation of large amounts of 13-methyl erythromycins which for the first time has permitted us to carry out a wide range of chemical modifications which had only been previously possible

15   starting from the "natural" erythromycins.

Some embodiments of the invention will now be described with reference to the accompanying drawings in which:

20   Fig 1 is a diagram showing the functioning of 6-deoxyerythronolide B synthase (DEBS), a modular PKS producing 6-deoxyerythronolide B (6-DEB) a precursor of erythromycin A.

Fig 2 gives the amino acid sequence comparison of the

25   KS domains and the CLF domains of representative Type II PKS gene clusters. The active site Cysteine (C) of the KS domains is arrowed in the Figure and aligns with the Glutamine (Q) or glutamic acid (E) of the CLF domains. The

abbreviations used, and the relevant Genbank/EMBL accession numbers are: GRA: granaticin from *Streptomyces violaceoruber* (X63449); HIR: unknown polyketide from *Saccharopolyspora hirsuta* (M98258); ACT, actinorhodin from

5 *Streptomyces coelicolor* (X63449); CIN: unknown polyketide from *Streptomyces cinnamonensis* (Z11511); VNZ: jadomycin from *Streptomyces venezuelae* (L33245); NOG: anthracyclines from *Streptomyces nogalater* (Z48262); TCM: tetracenomycin from *S. glaucescens* (M80674); DAU: daunomycin from

10 *Streptomyces sp.* C5 (L34880); PEU, doxorubicin from *Streptomyces peucetius* (L35560); WHI: WhiE spore pigment from *Streptomyces coelicolor* (X55942).

Fig 3 shows the gene organisation of the PKSs for three 16-membered ring macrolides, tylosin, spiramycin and

15 niddamycin.

Fig 4 shows the amino acid sequence alignment of KSq-ATq loading didomains of the PKSs for niddamycin, platenolide(spiramycin), monensin, oleandomycin and tylosin. The sequences for the monensin and oleandomycin

20 loading didomains have not been previously disclosed.

Fig. 5 The enyzmatic steps that convert 6-deoxyerythronolide B into erythromycin A *in Saccharopolyspora erythraea*

Fig. 6 is a diagram showing the construction of

25 plasmid pJLK117.

Fig. 7 shows the structures of two oligonucleotides.

The present invention will now be illustrated, but is

not intended to be limited, by means of some examples.

All NMR spectra were measured in $CDCl_3$ using a Bruker 500MHz DMX spectrometer unless otherwise indicated and peak positions are expressed in parts per million (ppm) downfield from tetramethysilane. The atom number shown in the NMR structure is not representative of standard nomenclature, but correlates NMR data to that particular example.

HPLC methods

Method A

Column	Waters Symmetry 5_ C18 2.1mm X 150 mm	
Flow	0.29 ml/min	
Mobile phase	Gradient: A:B (22:78) to A:B (38:62) over 12 minutes, then to A:B (80:20) by minute 15. Maintain for 1 minute. Re-equilibrate before next sample. Where A = acetonitrile and B = 0.01M ammonium acetate in 10% acetonitrile and 0.02% TFA	
Instrument	Acquired with Hewlett-Packard 1050 liquid chromatograph interfaced to a VG Platform II mass spectrometer equipped with an APCI source	

Method B
Column   Waters Symmetry 5_ C18 2.1mm X 150 mm
Flow 0.29 ml/min
Mobile phase   Gradient:28:72 acetonitrile:10mM NH4OAc to 50:50 in 18 minutes. 50:50 until 25 minutes. Back to 28:72, re-equilibrate for 7 minutes
Instrument   Acquired with Hewlett Packard 1100 LC/MS with APCI source

Tap Water medium

glucose	5g/liter
tryptone	5g/liter
yeast extract	2.5g/liter
EDTA	36mg/liter
Tap water to 1L total volume	

ERY - P medium

dextrose	50g/liter
Nutrisoy ™ flour	30g/liter
$(NH_4)_2SO_4$	3g/liter
NaCl	5g/liter
$CaCO_3$	6g/liter
Tap water to 1L total volume	
pH adjusted to 7.0	

Example 1

Construction of the Recombinant Vector pPFL43

Plasmid pCJR24 was prepared as described in PCT/GB97/01819. pPFL43 is a pCJR24-based plasmid containing the gene encoding a hybrid polyketide synthase that contains the putative monensin PKS loading module (isolated from *S. cinnamonensis*) the DEBS extension modules 1 and 2 and the chain-terminating thioesterase. Plasmid pPFL43 was constructed as follows:

The following synthetic oligonucleotides: 5'-CCATATGGCCGCATCCGCGTCAGCGT-3' and 5'-GGCTAGCGGGTCCTCGTCCGTGCCGAGGTCA-3' are used to amplify the DNA encoding the putative monensin-producing loading module using a cosmid that contains the 5' end of the putative monensin-producing PKS genes from *S. cinnamonensis* or chromosomal DNA of *S. cinnamonensis* as template. The PCR product of 3.3 kbp is purified by gel electrophoresis, treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which has been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL40. Plasmid pPFL40 was identified by restriction pattern and sequence analysis.

Plasmid pHD30His is a derivative of pNEWAVETE (PCT/GB97/01810) which contains the avermectin loading module, erythromycin extension modules 1 and 2 and the ery thioesterase domain. Plasmid pNEWAVETE was cut with

5   EcoRI and HinDIII and a synthetic oligonucleotide linker was inserted that encodes the addition of a C-terminal polyhistidine tail to the polypeptide. The following oligonucleotides:

5'-AATTCACATCACCATCACCATCACTAGTAGGAGGTCTGGCCATCTAGA-3'

10   and

5'-AGCTTCTAGATGGCCAGACCTCCTACTAGTGATGGTGATGGTGATGTG-3' were annealed together and the duplex was ligated to EcoRI-and HinDIII-cut pNEWAVETE. The resulting plasmid was cut with NdeI and XbaI and ligated into plasmid

15   pCJR24 that had been previously cut with same two enzymes, to produce plasmid pND30His.

Plasmid pPFL40 was digested with *Nde* I and *Nhe* I and the 3.3 kbp fragment was purified by gel electrophoresis and ligated to pND30-His previously digested with *Nde* I

20   and *Nhe* I and treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL43. Plasmid pPFL43 was identified by restriction analysis.

25

Example 2

Construction of *S. erythraea* NRRL2338/pPFL43

Plasmid pPFL43 was used to transform *S. erythraea*
NRRL2338 protoplasts. Thiostrepton resistant colonies
were selected in R2T20 medium containing 10 µg/ml of
thiostrepton. Several clones were tested for the
5   presence of pPFL43 integrated into the chromosome by
Southern blot hybridisation of their genomic DNA with
DIG-labelled DNA containing the *mon* PKS fragment
encoding for the loading module. A clone with an
integrated copy of pPFL43 was selected in this way.

10   **Example 3**

Production of 13-methyl-erythromycin A and B using *Sacch.*
***erythraea* NRRL 2338/pPFL43**

The culture *Saccharopolyspora erythraea*
NRRL2338(pPFL43), constructed with the wild-type loading
15   domain displaced by a monensin loader-D1TE DNA insert,
produced as described in Example 2, was inoculated into
30ml Tap Water medium with 50 ug/ml thiostrepton in a 300ml
Erlenmeyer flask. After three days incubation at 29°C, this
flask was used to inoculate 300 ml of ERY-P medium in a 300
20   ml flask. The broth was incubated at 29°C at 200 rpm for 6
days. After this time, the whole broth was adjusted to pH
8.5 with NaOH, then extracted with equal volume of ethyl
acetate. The ethyl acetate extract was evaporated to
dryness at 45°C under a nitrogen stream using a Zymark
25   TurboVap LV Evaporator, then reconstituted in 0.0625
volumes methanol to concentrate the extract 16-fold. The
structures of the products were confirmed by LC/MS, Method
A. A 4.0 min retention time peak was observed as the major
component, with *m/z* value of 720 $(M+H)^+$, required for 13-
30   methyl-erythromycin A. A second peak was observed with a
retention time of 6.4 min and with *m/z* value of 704 $(M+H)^+$,
required for 13-methyl-erythromycin B.

Example 4
35   **Production and Recovery of 13-methyl-erythromycin A and B**
using *Sacch. erythraea* NRRL-2338 (pPFL43) at 8L scale

Saccharopolyspora erythraea NRRL2338 (pPFL43) was
inoculated into 1000mls Tap Water medium with 50 µg/ml

thiostrepton in a 2.8l Fernbach flask. After three days incubation at 29°C, this flask was used to inoculate 8l of ERY-P medium in a 14l Microferm fermentor jar (New Brunswick Scientific Co., Inc., Edison, NJ). The broth was
5   incubated at 28°C with an aeration rate of 8l/min, stirring at 800 rpm and with pH maintained between 6.9 and 7.3 with NaOH or H_2SO_4 (15%). Water was added to maintain volume at the 24 hour volume level. The fermentation was continued for 167 hours. After this time, presence of 13-methyl-
10   erythromycin A and B were confirmed by adjusting a broth sample from the fermentor to pH 8.5 with NaOH, then extracting with equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then
15   reconstituted in 0.25 volumes methanol to concentrate the extract 4-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.1 min retention time peak was observed as the major component, with m/z value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A. A second
20   peak was observed with a retention time of 6.6 min and with m/z value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B.

     About 35 liters of broth containing approximately 2.8 grams of 13-methyl- erythromycin A were processed for recovery
25   of product. Broth was filtered through a pilot sized Ceraflo ceramic unit and loaded onto a 500ml XAD-16 resin column. The product was eluted using 100% methanol. A 175ml CG-161 adsorption column was prepared and equilibrated with 20% methanol/water. A portion of the product solution was adjusted
30   to 20% methanol and loaded onto the column, no breakthrough of product was observed. Washing of the column with up to 40% methanol/water failed at removing any significant level of impurities. Elution with 50% methanol/water achieved chromatographic separation of the product from the two major
35   impurities, 13-methyl-erythromycin B and a degradation product, 13-methyl-dehydroerythromycin A. The purest cuts were combined and reduced in volume by approximately 75% using evaporation to achieve <10% methanol concentration. To enhance 13-methyl-erythromycin A extraction, solid sodium bicarbonate
40   was added until a total concentration of 250mM was obtained. The aqueous product layer was extracted 2x with methylene chloride, using one-half the total volume each time. The volume was reduced to light yellow solids by evaporation. The 13-methyl-erythromycin A was purified by dissolving the crude
45   crystals into methylene chloride at ambient temperature and diluting to 15% methylene chloride with hexane. The cloudy solution is placed at -10°C for ~30 minutes when the liquid is decanted to a 2nd flask, leaving the majority of impurities behind as an oil. The flask is left overnight at -10°C,
50   followed by filtration of off-white13-methyl-erythromycin A crystals the next day. Approximately 300 milligrams of 13-methyl-erythromycin A were isolated from the partial work-up of the 35l broth volume.

Approximately 100 grams of evaporated mother liquor were utilized further to isolate 13-methyl-erythromycin B. Residual 13-methyl-erythromycin A was removed with repetitive extraction of the initial sample with aqueous acetic acid (pH 5). The subsequent methylene chloride layer was chromatographed on 700 g of silica gel using 20% methanol in methylene chloride. The 13-methyl-erythromycin B enriched fractions, as determined by LC/MS, were combined and evaporated to yield ~11.0 grams of dark oil. The oil was dissolved in a minimal amount of methanol and loaded onto 500 ml of Amberchrom CG-161 resin. The 13-methyl-erythromycin B was eluted at 2 bed volumes per hour with 40% methanol in deionized water. One bed volume fractions were collected and assayed by LC/MS. Fractions 42 through 62 were combined, diluted to ~20% methanol with deionized water, and neutralized to pH 7.5 with sodium bicarbonate. The resulting solution was extracted once with 41 of methylene chloride, concentrated to ~500 ml, and dried over anhydrous magnesium

sulfate. After removal of the MgSO4 by filtration the filtrate was evaporated to give ~110 mg of light brown solids. The 110 mg of crude 13-methyl-erythromycin B was dissolved in ~ 3.0 milliliters of HPLC grade acetonitrile and loaded onto a 20cm x 20cm, 2mm thick, silica gel preparative thin layer chromatography (PTLC) plate. The plate was developed with 60:40 methanol:acetonitrile. The desired portion of silica from the PTLC plate (iodine visualisation) was removed and extracted with HPLC grade acetone. The acetone extract was evaporated to give 12.1 mg of clear solid.

Identification of the 13-methyl-erythromycin A and 13-methyl-erythromycin B samples were confirmed by mass spectroscopy (LC/MS Method B) and NMR spectroscopy. The 13-methyl-erythromycin A sample peak had a 4.7 min retention time, with m/z value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A. The 13-methyl-erythromycin B sample peak had a 7.6 min retention time, with m/z value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B.

NMR, 13-methyl-erythromycin A:
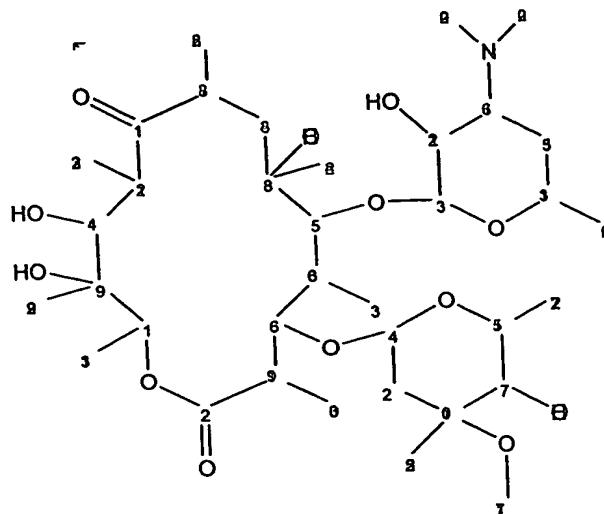
#	13C - ppm	#H	1H - ppm
1	221.91	0	
2	175.99	0	
3	103.63	1	4.45
4	96.81	1	4.88
5	83.76	1	3.60
6	79.86	1	4.10
7	78.36	1	3.05
8	75.50	0	
9	74.87	0	
10	73.07	0	
11	72.25	1	5.19
12	71.25	1	3.26
13	69.53	1	3.53
14	69.24	1	3.97
15	66.16	1	4.06
16	65.96	1	2.48
17	49.96	3	3.36
18	45.36	1	2.79
19	45.07	1	2.81
20	40.73	3	2.32
21	39.00	1	3.15
22	35.30	2	2.42/1.61
24	27.20	3	1.50
25	21.92	3	1.28
26	21.82	3	1.27
27	18.99	3	1.32
28	18.60	3	1.22
29	16.07	3	1.19
30	15.08	3	1.19
31	14.23	3	1.26
32	12.12	3	1.19

33	9.60	3	1.15
34	39.00	2	1.98/1.75
35	28.90	2	1.72/1.27
36	40.94	1	2.05

5

NMR, 13-methyl-erythromycin B:
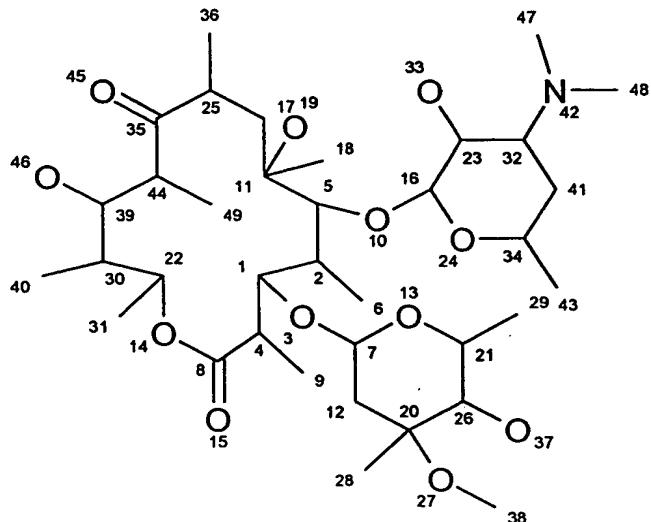
10

#	13C - PPM	#H attached	1H - PPM
1	80.50	1	4.15
2	40.62	1	2.15
4	45.17	1	2.84
5	84.08	1	3.62
6	9.86	3	1.18
7	97.26	1	4.88
8	176.48	0	
9	15.25	3	1.22
11	75.98	0	
12	35.43	2	2.42/1.61
16	103.75	1	4.46
17	38.77	2	2.09/1.72
18	27.67	3	1.51
20	73.09	0	
21	66.20	1	4.06
22	70.27	1	5.58
23	71.24	1	3.28
25	45.49	1	2.81

15

20

25

26	78.29	1	3.06
28	21.91	3	1.28
29	19.03	3	1.33
30	41.61	1	1.65
31	18.73	3	1.29
32	65.94	1	2.53
34	69.52	1	3.55
35	219.92	0	
36	19.03	3	1.21
38	49.97	3	3.36
39	70.17	1	3.88
40	9.27	3	0.95
41	29.12	2	1.73/1.28
43	21.80	3	1.27
44	39.87	1	3.07
47	40.74	3	2.35
48	40.74	3	2.35
49	9.62	3	1.04

Example 5
Construction of plasmid pPFL35

, Plasmid pPFL35 is a pCJR24-based plasmid containing a PKS gene comprising a loading module, the first and second extension modules of DEBS and the chain terminating thioesterase. The loading module comprises the KSq domain DNA from the loading module of the oleandomycin PKS fused to the malonyl-CoA-specific AT of module 2 of the rapamycin PKS, in turn linked to the DEBS loading domain ACP. Plasmid pPFL35 was constructed via several intermediate plasmids as follows:

A 411 bp DNA segment of the *ery*AI gene from *S.erythraea* extending from nucleotide 1279 to nucleotide 1690 (Donadio, S. *et al.*, Science (1991) 2523:675-679) was amplified by PCR using the following synthetic oligonucleotide primers:-

5'-TGGACCGCCGCCAATTGCCTAGGCGGGCCGAACCCGGCT-3' and

5'-CCTGCAGGCCATCGCGACGACCGCGACCGGTTCGCC-3'

The DNA from a plasmid designated pKSW, derived from pT7-7 and DEBS1-TE in which new *Pst* I and *Hind*III sites had been introduced to flank the KS1 of the first extension module, was used as a template. The 441 bp PCR product was treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL26. The new *Mfe* I/*Avr* II sites

bordering the insert are adjacent to the *Eco* RI site in the polylinker of pUC18. Plasmid pPFL26 was identified by restriction pattern and sequence analysis.

An *Mfe* I restriction site is located 112 bp from the
5   5' end of the DNA encoding the propionyl-CoA:ACP transferase of the loading module of DEBS. Plasmid pKSW was digested with *Mfe* I and *Pst* I and ligated with the 411 bp insert obtained by digesting plasmid pPFL26 with *Mfe* I and *Pst* I. The ligation mixture was used to transform
10   electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL27. Plasmid pPFL27 contains a PKS gene comprising the DEBS loading module, the first and second extension modules of DEBS and the DEBS chain terminating thioesterase. Plasmid pPFL27 was
15   identified by its restriction pattern.

Plasmid pPFL27 was digested with *Nde* I and *Avr* II and ligated to a 4.6kbp insert derived from digesting plasmid pMO6 (PCT/GB97/01819) with *Nde* I and *Avr* II. Plasmid pMO6 contains a PKS gene comprising the DEBS loading module, the
20   first and second extension modules of DEBS and the DEBS chain terminating thioesterase, except that the DNA segment encoding the methylmalonate-specific AT within the first extension module has been specifically substituted by the DNA encoding the malonate-specific AT of module 2 of the
25   *rap* PKS. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL28. Plasmid pPFL28 contains a hybrid PKS gene comprising the DEBS

loading module, the malonate-specific AT of module 2 of the *rap* PKS, the ACP of the DEBS loading module, followed by the first and second extension modules of DEBS and the DEBS chain terminating thioesterase. Plasmid pPFL28 was identified by restriction analysis.

A DNA segment encoding the KSq domain from the *ole*AI gene of *S. antibioticus* extending from nucleotide 1671 to nucleotide 3385 was amplified by PCR using the following synthetic oligonucleotide primers:-

5'-CCACATATGCATGTCCCCGGCGAGGAA-3' and

5'-CCCTGTCCGGAGAAGAGGAAGGCGAGGCCG-3'

and chromosomal DNA from *Streptomyces antibioticus* as a template. The PCR product was treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL31. The new *Nde* I site bordering the insert is adjacent to the *Eco* RI site of the pUC18 polylinker while the new *Bsp* EI site borders the *Hin* dIII site of the linker region. Plasmid pPFL31 was identified by restriction and sequence analysis.

Plasmid pPFL31 was digested with *Nde* I and *Avr* II and the insert was ligated with plasmid pPFL28 that had been digested with *Nde* I and *Avr* II. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and

individual clones were checked for the desired plasmid, pPFL32. Plasmid pPFL32 was identified by restriction analysis.

Plasmid pPFL32 was digested with *Nde* I and *Xba* I and the insert was ligated to plasmid pCJR24, which had been digested with *Nde* I and *Xba* I and purified by gel electrophoresis. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL35. Plasmid pPFL35 was identified by restriction analysis.

Example 6

Construction of *S. erythraea* NRRL2338/pPFL35

Plasmid pPFL35 was used to transform *S.erythraea* NRRL2338 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium (Yamamoto *et al.*) containing 10 µg/ml of thiostrepton. Several clones were tested for the presence of pPFL35 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the *rap* PKS fragment encoding for module 2 AT. A clone with an integrated copy of pPFL35 was identified in this way.

Example 7

Production of 13-methyl-erythromycin A and B using *Sacch. erythraea* NRRL-2338 (pPFL35)

The culture *Saccharopolyspora erythraea* NRRL2338(pPFL35), constructed with the wild-type loading

domain displaced by an oleandomycin KSQ-rapamycin AT2-D1TE DNA insert, prepared as described in Example 6, was inoculated into 30ml Tap Water medium with 50 ug/ml thiostrepton in a 300ml Erlenmeyer flask. After two days

5   incubation at 29°C, this flask was used to inoculate 300 ml of ERY-P medium in a 300 ml flask. The broth was incubated at 29°C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with an equal volume of ethyl acetate. The ethyl acetate extract

10   was evaporated to dryness at 45°C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then reconstituted in 0.25 volumes methanol to concentrate the extract 4-fold. The structures of the products were confirmed by LC/MS, Method A. A peak was observed with a retention time of 4.0

15   min and with an m/z value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A $(C_{36}H_{65}NO_{13})$. A second peak was observed with a retention time of 6.4 min and with m/z value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B $(C_{36}H_{65}NO_{12})$.

20   **Example 8**

Construction of Recombinant Vector pPFL44

Plasmid pPFL44 is a pCJR24- based plasmid containing the gene encoding a hybrid polyketide synthase that contains the spiramycin PKS loading module, the

25   erythromycin extension modules 1 and 2 and the chain-terminating thioesterase. Plasmid pPFL44 was constructed as follows:

The following synthetic oligonucleotides:

30   5'-CCATATGTCTGGAGAACTCGCGATTTCCCGCAGT-3' and

5'-GGCTAGCGGGTCGTCGTCGTCCCGGCTG-3'

were used to amplify the DNA encoding the spiramycin-producing loading module using chromosomal DNA from the spiramycin producer *S. ambofaciens* prepared according to

35   the method described by Hopwood *et al.* (1985). The PCR

product of 3.3 kbp was purified by gel electrophoresis,

treated with T4 polynucleotide kinase and ligated to

plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones

5   were checked for the desired plasmid pPFL41. Plasmid pPFL41 was identified by restriction pattern and sequence analysis.

Plasmid pPFL41 was digested with *Nde* I and *Nhe* I and the

10 3.3 kbp fragment was purified by gel electrophoresis and ligated to pND30 (a plasmid derived from plasmid pCJR24 having as insert the *ave* PKS loading module and extension modules 1 and 2 or DEBS and the DEBS thioesterase) (PCTGB97/01810) previously digested with *Nde* I and *Nhe* I

15 and treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones checked for the desired plasmid pPFL44. Plasmid pPFL44 was identified by restriction analysis.

20

Example 9

Construction of *Sacch. erythraea* NRRL2338/pPFL44

Plasmid pPFL44 was used to transform *S.erythraea* NRRL2338 protoplasts. Thiostrepton resistant colonies

25 were selected in R2T20 medium containing 10 µg/ml of thiostrepton. Several clones were tested for the presence of pPFL44 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA

containing the spiramycin PKS fragment encoding for the loading module. A clone with an integrated copy of pPFL44 was identified in this way.

5 **Example 10**
Production of 13-methyl-erythromycin A and B using *Sacch. erythraea* NRRL-2338 (pPFL44)

The culture *Saccharopolyspora erythraea* NRRL2338
10 (pPFL44), constructed with the wild-type loading domain displaced by spiramycin loader-D1TE DNA insert, was inoculated into 30ml Tap Water medium with 50 ug/ml thiostrepton in a 300ml Erlenmeyer flask. After three days incubation at 29°C, this flask was used to inoculate 300 ml
15 of ERY-P medium in a 300 ml flask. The broth was incubated at 29°C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream
20 using a Zymark TurboVap LV Evaporator, then reconstituted in 0.0625 volumes methanol to concentrate the extract 16-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.0 min retention time peak was observed as the major component, with *m/z* value of 720 $(M+H)^+$,
25 required for 13-methyl-erythromycin A $(C_{36}H_{65}NO_{13})$. A second peak was observed with a retention time of 6.4 min and with *m/z* value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B $(C_{36}H_{65}NO_{12})$.

30 **Example 21**

Construction of plasmid pJLK114

Plasmid pJLK114 is a pCJR24 based plasmid containing a
35 PKS gene comprising the ery loading module, the first and the second extension modules of the ery PKS and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module

has been substituted by a synthetic oligonucleotide
linker containing the recognition sites of the following
restriction enzymes: AvrII, BglII, SnaBI, PstI, SpeI,
NsiI, Bsu36I and HpaI. It was constructed via several
intermediate plasmids as follows (Figure 6).

Construction of plasmid pJLK02

The approximately 1.47 kbp DNA fragment of the eryAI gene
of S. erythraea was amplified by PCR using as primers the
synthetic oligonucleotides:
5'-TACCTAGGCCGGGCCGGACTGGTCGACCTGCCGGGTT-3' and
5'-ATGTTAACCGGTCGCGCAGGCTCTCCGTCT-3' and plasmid pNTEP2
(Oliynyk, M. et al., Chemistry and Biology (1996) 3:833-
839; WO98/01546) as template. The PCR product was treated
with T4 polynucleotide kinase and then ligated with
plasmid pUC18, which had been linearised by digestion
with SmaI and then treated with alkaline phosphatase. The
ligation mixture was used to transform electrocompetent
E. coli DH10B cells and individual colonies were checked
for their plasmid content. The desired plasmid pJLK02 was
identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK03

The approximately 1.12 kbp DNA fragment of the eryAI gene
of S. erythraea was amplified by PCR using as primers the
synthetic oligonucleotides:
5'-ATGTTAACGGGTCTGCCGCGTGCCGAGCGGAC-3' and

5'-CTTCTAGACTATGAATTCCCTCCGCCCAGC-3' and plasmid pNTEPH as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK03 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK04

Plasmid pJLK02 was digested with PstI and HpaI and the 1.47 kbp insert was ligated with plasmid pJLK03 which had been digested with PstI and HpaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK04 was identified by its restriction pattern.

Construction of plasmid pJLK05

Plasmid pJLK01 (PCT/GB97/01819) was digested with PstI and AvrII and the 460 bp insert was ligated with plasmid pJLK04 which had been digested with PstI and AvrII. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK05 was identified by its restriction pattern.

Construction of plasmid pJLK07

Plasmid pJLK05 was digested with ScaI and XbaI and
plasmid pNTEPH was digested with NdeI and ScaI and these
5   two fragments were ligated with plasmid pCJR24 which had
been digested with NdeI and XbaI. The ligation mixture
was used to transform electrocompetent E. coli DH10B
cells and individual colonies were checked for their
plasmid content. The desired plasmid pJLK07 was
10   identified by its restriction pattern.

Construction of plasmid pJLK114

The two synthetic oligonucleotides P1f and P1b (Figure 7)
15   were each dissolved in TE-buffer. 10 μl of each solution
(0.5nmol/μl) were mixed and heated for 2 minutes to 65C
and then slowly cooled down to room temperature. Plasmid
pJLK07 was digested with AvrII and HpaI and ligated with
the annealed oligonucleotides. The ligation mixture was
20   used to transform electrocompetent E. coli DH10B cells
and individual colonies were checked for their plasmid
content. The desired plasmid pJLK114 was identified by
its restriction pattern.

25   Plasmid pJLK117 is a pCJR24 based plasmid containing a
PKS gene comprising the ery loading module, the first and
the second extension modules of the ery PKS and the ery
chain-terminating thioesterase except that the DNA
segment between the end of the acyltransferase and the

beginning of the ACP of the second ery extension module has been substituted by a synthetic oligonucleotide linker containing the recognition sites of the following restriction enzymes. AvrII, BglII, SnaBI, PstI, SpeI,

5   NsiI, Bsu36I and NheI.

It was constructed via several intermediate plasmids as follows (Figure 6).

10 Construction of plasmid pJLK115

Plasmid pJLK114 was digested with NdeI and XbaI and the approximately 9.9 kbp insert was ligated with plasmid pUC18 which had been digested with NdeI and XbaI. The

15 ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK115 was identified by its restriction pattern.

20 Construction of plasmid pJLK116

Plasmid pJLK13 (PCT/GB97/01819) was digested with Bsu36I and XbaI and the 1.1 kbp fragment was ligated with plasmid pJLK115 which had been digested with Bsu36I and

25 XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK116 was identified by its restriction pattern.

30

Construction of plasmid pJLK117

Plasmid pJLK116 was digested with NdeI and XbaI and the
9.9 kbp fragment was ligated with plasmid pCJR24 which
5   had been digested with NdeI and XbaI. The ligation
mixture was used to transform electrocompetent E. coli
DH10B cells and individual colonies were checked for
their plasmid content. The desired plasmid pJLK117 was
identified by its restriction pattern.
10

Example 11

Construction of plasmid pJLK29

15   Plasmid pJLK29 is a pJLK117 based plasmid except that the
DNA fragment encoding the reductive loop of module 10 of
the rap PKS has been inserted into the mcs. It was
constructed via several intermediate plasmids as follows.
(Figure 5)
20

Construction of plasmid pJLK121.1

The approximately 2.2 kbp DNA segment of the rapB gene of
S. hygroscopicus encoding the reductive loop of module 10
25   was amplified by PCR using as primers the synthetic
oligonucleotides:

5'-TAAGATCTTCCGACGTACGCGTTCCAGC-3' and

5'-ATGCTAGCCACTGCGCCGACGAATCACCGGTGG-3' and as template
an approximately 7 kbp fragment, which has been obtained

by digestion of cosmid cos 26 (Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843) with ScaI and SphI. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had

5   been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK121.1 was identified by

10   its restriction pattern and DNA sequencing.

Construction of plasmid pJLK29

Plasmid pJLK121.1 was digested with BglII and NheI and

15   the 2.2 kbp fragment was ligated with plasmid pJLK117 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK29 was

20   identified by its restriction pattern.

Example 24

Construction of Plasmid pJLK50

25

The approximately 6.1 kbp DNA segment of the erythromycin PKS gene cluster of S. erythraea encoding the DNA fragment from the beginning of the ACP of module 2 to the beginning of the ACP of module 3 was amplified by PCR

using as primers the synthetic oligonucleotides:

5'-TACCTGAGGGACCGGCTAGCGGGTCTGCCGCGTG-3' and

5'-ATGCTAGCCGTTGTGCCGGCTCGCCGGTCGGTCC-3' and plasmid

pBAM25 (published pBK25 by Best, D J et al. Eur J Biochem

5   (1992) 204: 39-49) as template. The PCR product was

treated with T4 polynucleotide kinase and then ligated

with plasmid pUC18, which had been linearised by

digestion with SmaI and then treated with alkaline

phosphatase. The ligation mixture was used to transform

10   electrocompetent E. coli DH10B cells and individual

colonies were checked for their plasmid content. The

desired plasmid pJLK50 was identified by its restriction

pattern and DNA sequencing.

15   **Example 25**

Construction of *S.erythraea* strain JLK10

Strain JLK10 is a variant of strain NRRL2338 in which the

reductive loop of *ery* module 2 (i.e. the KR domain) is

20   replaced by the reductive loop of the rapamycin module

10. It was constructed using plasmid pJLK54 which was

constructed as follows.

Construction of plasmid pJLK54

25

Plasmid pJLK54 is a pJLK29 based plasmid containing a PKS

gene comprising the ery loading module, the first, the

second and the third extension modules of the ery cluster

and the ery chain-terminating thioesterase except that

the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by the equivalent segment of module 10 of the rapamycin PKS.

5   It was constructed as follows.

Plasmid pJLK50 was digested with NheI and the 6.1 kbp insert was ligated with plasmid pJLK29 which had been digested with NheI. The ligation mixture was used to
10   transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK54 was identified by its restriction pattern.

15   Use of plasmid pJLK54 for construction of S. erythraea NRRL2338/pJLK54 and the production of TKL derivatives

Approximately 5 μg plasmid pJLK54 were used to transform protoplasts of S. erythraea NRRL2338 and stable
20   thiostrepton resistant colonies were isolated. From several colonies total DNA is obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE.

25   **Construction of S.erythraea strain JLK10 and its use in production of 13-methyl-10,11-dehydro-erythromycin A**

S. erythraea strain JLK10 is a mutant of S. erythraea NRRL2338 in which the 'reductive loop' of ery module 2 i.e. the ketoreductase domain is substituted by the

'reductive loop' of rapamycin module 10. It was
constructed starting from S. erythraea NRRL2338 into
which plasmid pJLK54 had been integrated. S. erythraea
NRRL2338/pJLK54 was subjected to several rounds of non-
5 selective growth which resulted in second crossover
concomitant with the loss of the integrated plasmid.
Clones in which replacement of the erythromycin gene
coding for DEBS1 with the mutant version had occurred,
were identified by Southern blot hybridisation. One of
10 these was named S. erythraea strain JLK10 and was used to
inoculate SM3 medium (eryP medium gave similar results),
and allowed to grow for seven to ten days at 28-30°C.
After this time the broth was centrifuged and the pH of
the supernatant adjusted to pH 9. The supernatant was
15 then extracted three times with an equal volume of ethyl
acetate and the solvent was removed by evaporation.
Products were analysed by HPLC/MS, MS/MS and 1H-NMR. The
following macrolide C-13 methyl erythromycin A was
identified (accompanied by products of incomplete
20 processing by post-PKS enzymes).

25
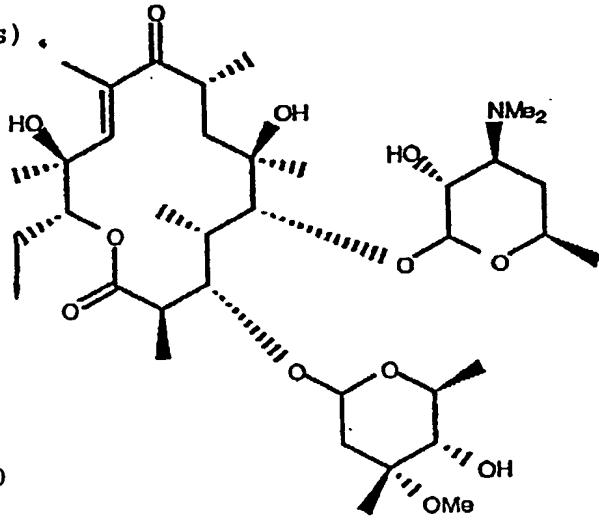
Example 26

Construction of plasmid pPFL50

Plasmid pPFL50 is a pPFL43-based plasmid from which a DNA

fragment encoding KR1 (in part), ACP1 and module 2 of the erythromycin PKS and the erythromycin TE, has been removed. It was constructed as follows. Plasmid pPFL43 was digested with SfuI and XbaI to remove a 6.5 kb

5   fragment. The 5' overhangs were filled in with Klenow fragment DNA Polymerase I and the plasmid was recircularised. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid

10 content. The desired plasmid pPFL50 was identified by its restriction pattern.

Construction of S. erythraea JLK10/pPFL50

Approximately 5 μg plasmid pPFL50 were used to transform

15 protoplasts of S. erythraea strain JLK10 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid had integrated into the homologous chromosomal DNA

20 region. S. erythraea strain JLK10/pPFL50 was used to inoculate SM3 medium containing 5 μg/ml thiostrepton (eryP medium containing 5 μg/ml thiostrepton gave similar results)and allowed to grow for seven to ten days at 28-30oC. After this time the broth was centrifuged and the

25 pH of the supernatant adjusted to pH 9. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by HPLC/MS, MS/MS and 1H-NMR. The macrolide C-13 methyl 10,11-dehydro-erythromycin A was

identified (accompanied by products of incomplete processing by post-PKS enzymes)

Construction of S. erythraea NRRL2338/pPFL50

5   Approximately 5 μg plasmid pPFL50 were used to transform protoplasts of S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid

10   had integrated into the homologous region of the chromosomal DNA. S. erythraea NRRL2338/pPFL50 was used to inoculate SM3 medium containing 5 μg/ml thiostrepton (eryP medium containing 5 μg/ml thiostrepton gives similar results) and allowed to grow for seven to ten

15   days at 28-30oC. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH 9.5. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by

20   HPLC/MS, MS/MS and 1H-NMR. The macrolide C-13 methyl erythromycin A was identified (accompanied by products of incomplete processing by post-PKS enzymes).

Construction of plasmid pCB121

25   Plasmid pCB121 is a plasmid containing the monensin loading module and KS of monensin module 1 followed by the erythromycin module 1 AT and part of the erythromycin module1 KR. It was constructed via several intermediate plasmids as follows.

Construction of plasmid pPFL45

The approximately 1.8 kbp DNA segment of the monensin PKS
gene cluster of Streptomyces cinnamonensis encoding part
of the ACP of the loading module and KS of module 1 was
amplified by PCR using as primers the synthetic
oligonucleotides:

5'-CGTTCCTGAGGTCGCTGGCCCAGGCGTA-3'

5'-CGAAGCTTGACACCGCGGCGCGGCGCGG-5'

and a cosmid containing the 5' end of the monensin PKS
genes from S. cinnamonensis or alternatively chromosomal
DNA of S. cinnamonensis as template. The PCR product was
treated with T4 polynucleotide kinase and then ligated
with plasmid pUC18, which had been linearised by
digestion with SmaI and then treated with alkaline
phosphatase. The ligation mixture was used to transform
electrocompetent E. coli DH10B cells and individual
colonies were checked for their plasmid content. The
desired plasmid pPFL45 was identified by its restriction
pattern.

Construction of plasmid pPFL47

Plasmid pPFL45 was digested with NdeI and Bsu36I and the approximately 2.6 kbp
fragment was ligated into plasmid pPFL43 which had been digested with NdeI and
Bsu36I. The ligation mixture was used to transform electrocompetent E. coli DH10B
cells and individual colonies were checked for their plasmid content. The desired
plasmid pPFL47 was identified by its restriction pattern.

Construction of plasmid pCB135

Plasmid pCJR24 was digested with HindIII, the 5' overhang
was filled in with Klenow fragment DNA Polymerase I and
religated. The ligation mixture was used to transform
electrocompetent E. coli DH10B cells and individual
colonies were checked for their plasmid content. The

desired plasmid pCB135 was identified by its restriction pattern, lacking the recognition site for HindIII.

Construction of plasmid pKSW1

Plasmid pKS1W is a pNTEP2 (GB97/01810)-derived vector containing a DEBS1TE-derived triketide synthase with the unique restriction sites introduced at the limits of KS1.

5   Plasmid pKS1W is obtained via several intermediate plasmids as follows.

Construction of plasmids pMO09, pMO10 and pMO13

For the PCR amplification for plasmid pMO09, the

10   following synthetic oligonucleotides were used as mutagenic primers, one containing a MunI site and the other a PstI site:

5' -GCGCGCCAATTGCGTGCACATCTCGAT- 3'

and 5' -CCTGCAGGCCATCGCGACGACCGCGACCGGTTCGCCG- 3'

15

For the PCR amplification for plasmid pMO10, the following synthetic oligonucleotides were used as mutagenic primers, one containing a HindIII site and the other an EcoRV site:

20   5' -GTCTCAAGCTTCGGCATCAGCGGCACCAA- 3'

and 5' -CGTGCGATATCCCTGCTCGGCGAGCGCA-3'

For the PCR amplification for plasmid pMO13, the following synthetic oligonucleotides were used as

25   mutagenic primers, one containing a PstI site and the other a HindIII site:

5' -GATGGCCTGCAGGCTGCCCGGCGGTGTGAGCA- 3'

and 5' -GCCGAAGCTTGAGACCCCCGCCCGGCGCGGTCGC- 3'

PCR was carried out on pNTEP2 (GB97/01810) as template using Pwo DNA polymerase and one cycle of: 96°C (1min); annealing at 50°C (3min); and extension at 72°C (1min), and 25 cycles of: 96°C (1min); annealing at 50°C (1min);

5   and extension at 72°C (1min) in the presence of 10% (vol/vol) dimethylsulphoxide. The products were end-repaired and cloned into pUC18 digested with SmaI and the ligation mixture was transformed into E. coli DH 10B. Plasmid DNA was prepared from individual colonies. The

10   desired plasmids for pMO09 (3.8kbp), pMO10 (3.9 kbp) and pMO13 (4.3 kbp) were identified by their restriction pattern and DNA sequencing.

Construction of plasmid pMO11

15   Plasmid pMO13 was digested with HindIII, and the 1.2 kbp insert was cloned into pMO10 which had been digested with HindIII. The ligation mixture was transformed into E. coli DH 10B. The desired plasmid (5.0 kbp) was identified by its restriction pattern and designated

20   pMO11.

Construction of plasmid pMO12

Plasmid pMO09 was digested with PstI, and the 1.6 kbp insert was cloned into pMO11 which had been digested with

25   PstI. The ligation mixture was transformed into E. coli DH 10B. The desired plasmid (6.6 kbp) was identified by its restriction pattern and designated pMO12.

Construction of pKS1W

Plasmid pMO12 was digested with MunI and EcoRV, and the 3.9 kbp fragment was cloned into pNTEPH (see below) which had been digested with MunI and EcoRV. The ligation mixture was transformed into E. coli DH 10B. The desired

5   plasmid (13. kbp) was identified by its restriction pattern and designated pKS1W.

Construction of pNTEPH

Plasmid pNTEPH was obtained from pNTEP2 by removing the

10 HindIII site. pNTEP2 was digested with HindIII, the 5' overhang was filled in with Klenow Fragment DNA Polymerase I and religated. The desired plasmid (13.6 kbp) was identified by its restriction pattern.

15 ## Construction of plasmid pCB136

Plasmid pKSW1 was digested with NdeI and XbaI and the approximately 11.2 kbp fragment was ligated with plasmid pCB135 which had been digested with NdeI and XbaI.The ligation mixture was used to transform electrocompetent

20 E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pCB136 was identified by its restriction pattern.

Construction of plasmid pCB137

25 Plasmid pCB136 was digested with SfuI and XbaI to remove a 6.5 kb fragment,. the 5' overhangs were filled in with Klenow Fragment DNA Polymerase I and religated. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked

for their plasmid content. The desired plasmid pCB137 was identified by its restriction pattern.

Construction of plasmid pCB121

5   Plasmid pPFL47 was digested with NdeI and HindIII and the approximately 4.4 kbp insert was ligated with plasmid pCB137 which had been digested with NdeI and HindIII. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked
10   for their plasmid content. The desired plasmid pCB121 was identified by its restriction pattern.

Example

Construction of S.erythraea JLK10/pCB121

15   Approximately 5 µg plasmid pCB121 were used to transform protoplasts of S. erythraea JLK10 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid had integrated
20   into the homologous chromosomal DNA region. S. erythraea strain JLK10/pCB121 was used to inoculate SM3 medium containing 5 µg/ml thiostrepton (eryP medium containing 5 µg/ml thiostrepton gave similar results) and allowed to grow for seven to ten days at 28-30oC. After this time
25   the broth was centrifuged and the pH of the supernatant adjusted to pH 9. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were

analysed by HPLC/MS, MS/MS and 1H-NMR. The macrolide C13-methyl-10,11-dehydro-erythromycin A was identified (accompanied by products of incomplete processing by post-PKS enzymes):

5

Example

Construction of S. erythraea NRRL2338/pCB121

Approximately 5 µg plasmid pCB121 were used to transform protoplasts of S. erythraea NRRL2338 and stable

10 thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid had integrated into the homologous chromosomal DNA region. S. erythraea NRRL2338/pPFL50 was used to

15 inoculate SM3 medium containing 5 µg/ml thiostrepton (eryP medium containing 5 µg/ml thiostrepton gave similar results) and allowed to grow for seven to ten days at 28-30oC. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH=9.. The supernatant

20 was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by HPLC/MS, MS/MS and 1H-NMR. The macrolide C13-erythromycin A was identified (accompanied by products of incomplete processing by post-PKS

25 enzymes):

Although the present invention is illustrated by the examples listed above, they should not be regarded as limiting the scope of the invention. The above

descriptions illustrate for the first time the
construction of a Type I PKS gene assembly containing a
wholly or partly heterologous KSq-containing loading
module and its use to obtain polyketide products of

5   utility as synthetic intermediates or as bioactive
materials such as antibiotics. It will readily occur to
the person skilled in the art that a wholly or partly
heterologous KSq-containing loading module from other PKS
gene sets could be used to replace the loading module of

10   DEBS, or indeed into a quite different PKS gene assembly.
It will also readily occur to the person skilled in the
art that that the additional specificity provided by the
more efficient discrimination made between methylmalonyl-
CoA and malonyl-CoA by an ATq, followed by specific

15   decarboxylation by a KSq, is preferable to the imperfect
discrimination between propionyl-CoA and acetyl-CoA that
is a feature of the DEBS loading module and of many other
PKS loading modules, in that it maximises the production
of a single product rather than a mixture differing from

20   each other in the nature of the starter unit. The
avoidance of such mixtures increases yields and avoids
the need for tedious and difficult separation procedures.